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Formalin-Fixed Paraffin-Embedded Clinical Tissues Show Spurious Copy Number Changes in Array-CGH Profiles

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Brief Title: **Array-CGH of FFPE tissues**

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Abstract

Formalin-fixed paraffin-embedded (FFPE) archival clinical specimens are invaluable in discovery of prognostic and therapeutic targets for diseases such as cancer. However, the suitability of FFPE-derived genetic material for array-based comparative genomic hybridisation (array-CGH) studies is under-explored. In this study, genetic profiles of matched FFPE and fresh-frozen specimens were examined to investigate DNA integrity differences between these sample types and determine the impact this may have on genetic profiles. Genomic DNA was extracted from three patient-matched FFPE and fresh-frozen clinical tissue samples. T47D breast cancer control cells were also grown in culture and processed to yield a fresh T47D sample, a fresh-frozen T47D sample and a FFPE T47D sample. DNA was extracted from all samples, array-CGH conducted and genetic profiles of matched samples were then compared. A loss of high-molecular weight DNA was observed in the FFPE clinical tissues and FFPE T47D samples, respectively. A dramatic increase in absolute numbers of genetic alterations was observed in all FFPE tissues relative to matched fresh-frozen counterparts. In future, alternative fixation and tissue processing procedures, and/or new DNA extraction and CGH profiling protocols, may be implemented, enabling identification of changes involved in disease progression through the use of stored clinical specimens.

Introduction

Genomic instability is a hallmark of human cancer [1]. Amplification or deletion of distinct sub-chromosomal areas can lead to over- or under-expression of key genes, thus conferring a growth advantage to malignant cells [2]. Conversely, deletions of tumour suppressor genes, such as those involved in cell death, may also push the genetic balance toward malignant growth. Amplified genes are therefore important targets for therapeutic invention, and identification of such copy number alterations can help elucidate potential mechanisms involved in tumour development and progression, and identify cancer subtypes.

The most widely used and successful technique to study copy number alterations (CNAs) is comparative genomic hybridisation (CGH). Array-CGH is conducted by differentially labelling total genomic DNA from test and reference samples, followed by co-hybridisation onto an array consisting of DNA probes spanning the genome. The ratio of fluorescent intensities, allows identification of copy number change between test and reference samples. Classically, this technique has been successfully used on DNA extracted from fresh-frozen clinical tissue specimens, as these yield higher quality nucleic acids [3-5]. However, as the availability of fresh-frozen tissue is often limited, studies of archival formalin-fixed paraffin-embedded (FFPE) clinical tissue specimens with accompanying retrospective data would be of enormous benefit in elucidating key genes involved in disease progression. Array-CGH studies using FFPE DNA have been reported [6-8], although little is known of the true impact of tissue fixation and processing on resulting genetic profiles.

The present study was primarily performed to assess the correlation of array-CGH profiles of DNA extracted from matched FFPE and fresh-frozen tissues, investigate the number of CNAs present in DNA extracted from FFPE tissues with respect to their matched fresh-frozen counterparts, and to assess any difference in DNA quality between these sample types.

Materials and Methods

Cell Line and Tissue Preparation

T47D breast cells (E.C.A.C.C., UK) were cultured in DMEM + 10% FBS, 200mM L-glutamine. Three tissue culture flasks were grown to confluency. Three identical cell T47D cell pellets were prepared and genomic DNA was immediately extracted from the first. A FFPE cell sample was prepared from the second pellet, by first fixing T47D cells by suspension in 10% neutral buffered formalin (NBF) for 1 hr at room temperature. The pellet was centrifuged, formalin removed, 70% ethanol added, mixed and left overnight. The pellet was then dehydrated through increasing concentrations of ethanol (70%, 95%, 100%, 100%) at room temperature for 1hr 45m each and then finally in xylene at room temperature for two steps of 1hr 45m each. Liquid paraffin was added to the pellet and cells were maintained at 60°C (melting point of paraffin) and replaced every 20m for a period of 2hr to ensure removal of residual xylene. The pellet was then allowed to solidify a room temperature in fresh paraffin and was then implanted in a new paraffin block from which sections were then cut for DNA extraction from FFPE cells. Fresh-frozen T47D cells were prepared by snap-freezing the third pellet of T47D cells in optimum cutting temperature (O.C.T.) matrix. The frozen block was stored at -80°C for one week, then trimmed to remove excess O.C.T matrix from the frozen cell pellet, and fresh-frozen DNA was extracted.

Three cases of patient-matched fresh-frozen and FFPE colon cancer tissue samples were also acquired for array-CGH investigation from the Department of Histopathology, Beaumont Hospital, Dublin 9. DNA was extracted from a 20µm section of each fresh-frozen tissue and each matched FFPE tissue, which had been

processed as follows. Colon cancer tissues for frozen storage were immediately frozen using liquid nitrogen upon reaching the histopathology laboratory from surgery. Samples were stored at -80°C for a maximum of 2 months prior to frozen sectioning on a cryostat. DNA was immediately extracted after sectioning. Matched colon cancer FFPE tissues were fixed in 10% formalin for an average of 16 hr before embedding in paraffin. Blocks were stored at room temperature for a maximum of 2 months prior to sectioning. DNA was then extracted within one week of sectioning.

DNA Extractions

DNA from all above sources was extracted using the NucleoSpin Tissue Kit (Clontech, USA). The following additional protocol was applied prior to DNA extraction from FFPE tissue. FFPE tissue sections were placed in 1.5ml tubes. Xylene (1ml) was added and mixed at room temperature for 30 min. Tubes were microcentrifuged for 3 min at 11,000 x g to pellet tissue, and subsequent supernatant removed. Ethanol (96% v/v; 1ml) was added to each tissue, mixed, and microcentrifuged for 3 min at 11,000 x g then ethanol was removed. This step was repeated, and after removal of ethanol the tissue was incubated at 37°C until all ethanol had evaporated. The standard NucleoSpin Tissue Kit manual procedure was then applied to all tissues for extraction of DNA. Extracted DNA was quantified using a NanoDrop ND-1000 Spectrophotometer (Nanodrop Technologies, USA). DNA integrity was assessed by analysis of 100ng DNA via 2% agarose gel electrophoresis.

Array-CGH

The GenoSensor array-CGH platform (Vysis - Abbott Laboratories, USA) was utilised in this study. In brief, test and reference DNA (100ng) were labelled with Cy3 and Cy5 fluorophores (1mM) respectively. Labelled targets were DNase-treated for 1 hr at 15°C then purified by sodium acetate and ethanol precipitation steps. The quality of purified, labelled DNA was assessed by agarose gel electrophoresis. Equal concentrations of labelled test and reference DNA were then combined and loaded onto the GenoSensor Array 300 array. Hybridisation was conducted in the presence of humidified 2X standard saline citrate (SSC)/50%(v/v) formamide for 60 hr at 37°C. Arrays were washed in 2X SSC/50%(v/v) formamide, followed by 1XSSC. Spots were counterstained with DAPI and scanning was performed using the GenoSensor Reader System.

Image Acquisition and Data Analysis

The GenoSensor reader was used to acquire a three-color image of each microarray using a CCD camera and a 175W xenon light source. Automated analysis was then conducted by the GenoSensor Reader software as follows. Spot segmentation and identification was conducted to isolate and relate every DNA spot on the microarray to a particular DNA target clone. Spot intensity and brightness was measured for each spot and ratios of Cy3 to Cy5 fluorescence were calculated. Ratio values were then normalized, subjected to statistical analysis using an analog of the t-test, and placed in a report format. This t-test analog takes account of modal target variability, replicate spot ratio variability of the target being assessed, and assumes a normal distribution of modal target mean ratios, thus providing an unbiased significance estimate for non-modality.

The resulting target report contained the mean and coefficient-of-variation of the normalized CGH ratios of the three replicate spots, together with a significance or p-

value that the mean ratio corresponds to a gain or loss of copy number for each target clone on the array. Only those targets whose computed p-values were less than 0.01 for all samples were selected in this study. This was conducted to improve the stringency of results and, thus, the comparability of resulting genetic profiles of alternative sample types. Subsequent target report tables from matched fresh-frozen and FFPE specimens were compared and numbers of genetic alterations and areas of genomic change were recorded. Additionally quality parameters, as set by the array manufacturers, were recorded for each array. Only those arrays passing all quality parameters were included in this study.

Results

This study was conducted to primarily investigate the possible impact of formalin-fixation and paraffin-embedding on DNA degradation and subsequent DNA profiling. Prior to profiling using array-CGH, we assessed possible differences in integrity of DNA extracted from FFPE and fresh-frozen matched samples by agarose gel electrophoresis using equal amounts of DNA. Genomic DNA extracted from matched FFPE and fresh-frozen clinical samples show dramatic differences in quality by agarose gel electrophoresis (**Figure 1**). Lanes 1-3 show a complete lack of high molecular weight DNA in FFPE clinical samples. Lanes 4-6 show the retention of high molecular weight DNA in fresh-frozen clinical samples, with minor degradation as indicated by the appearance of lower molecular weight DNA. DNA samples extracted from three alternatively-processed T47D breast cancer cell samples were also compared. Lane 8 shows the retention of high molecular weight DNA in the DNA extracted from fresh T47D cells. Lane 9 shows that snap-frozen O.C.T.-embedded T47D cells produced DNA of high molecular weight with little degradation. Lane 7 shows a large DNA smear, with the loss of the majority of high molecular weight DNA. This DNA, which was extracted from FFPE T47D cells, displayed the highest degradation of the three matched T47D samples. These results indicate that formalin-fixation and paraffin-embedding may have a negative effect on the quality of extracted DNA. DNA yields from differentially-processed pellets of 3.5 X 10^7 T47D cells also differed significantly. Yields of 43.2, 7, and 1.6ug were quantified for fresh, OCT fresh-frozen, 1hr formalin-fixed cells, respectively.

We next sought to identify any differences in the array-CGH profiles of DNA from alternatively-processed samples (**Figure 2**). A dramatic difference in the total number

of statistically-relevant genetic changes between matched FFPE and fresh-frozen samples was observed (**Figure 2a**). Of the additional genetic changes observed in all FFPE specimens relative to matched counterparts, there was an almost equal increase amplifications and deletions (**Figure 2b**). No pattern of alterations or chromosomal region bias was evident in these FFPE tissues, as alterations occurred on almost all chromosomes. Notably, an increase in the number of genetic alterations, from 28 to 122, was observed for fresh- and FFPE-derived DNA from identical T47D breast tumour cell line samples. The absolute numbers of genetic changes, as well as regions where these occurred, in the fresh T47D DNA sample correlated well with those previously reported in the literature using standard chromosome CGH spreads and spectral karyotyping (SKY) techniques [9,10].

Differences can also be observed when comparing array-CGH profiles of DNA extracted from the three alternatively fixed and processed T47D cell line-derived samples (**Figure 3a**). Profiles of fresh and snap-frozen samples are almost identical; however, a large increase in genetic changes can be seen in the FFPE DNA sample. We also sought to examine the difference in array-CGH profiles of alternatively processed clinical samples. It can be seen that the array-CGH profiles of all three FFPE clinical samples display an obvious increase in genetic changes relative to their patient-matched fresh counterparts **(Figure 3b)**.

Discussion

We have shown that suitable quantities of DNA for investigation using the GenoSensor array-CGH platform can be obtained from both snap-frozen and FFPE samples. Prior to conducting array-CGH, test genomic DNA was visualised by agarose gel electrophoresis to assess potential degradation. As expected from previously published studies [11-14], all FFPE samples showed an increase in degradation, and a loss of high molecular weight DNA in comparison to matched fresh-frozen specimens. A complete loss of high molecular weight DNA was observed in all FFPE clinical tissues examined, indicating a substantial deleterious effect of formalin fixation on DNA quality. In our hands, different DNA extraction kits had negligible effects on DNA quality after formalin fixation, producing similar highly degraded DNA (data not shown). Formalin fixation alone was shown to be sufficient to produce degraded DNA, which showed multiple spurious genetic alterations in array-CGH profiles (Supplementary Figures 1 and 2). Additionally array-CGH genetic profiles of clinical archival FFPE and fresh-frozen colon tissues showed a notable increase in total numbers of statistically relevant genetic alterations in FFPE tissues compared to patient-matched fresh-frozen specimens.

Previous reports have noted differences in DNA extracted from matched FFPE and fresh tissue specimens. Inoue *et al*. and Serth *et al*. both reported a reduction in the yield of total genomic DNA from FFPE specimens compared to matched frozen counterparts [12,14]. Discordances between array-CGH profiles of alternatively processed samples have also been reported, noting the appearance of spurious copy number gains and losses in FFPE material only. Lee et al. noted that matched FFPE and frozen samples exhibited similar array-CGH profiles, but also observed several

discordant aberrations in FFPE samples [15]. Little *et al.* reported the same phenomenon, citing tumour heterogeneity, alternate tissue sampling, or a drop in reliability of DNA extracted from FFPE tissue as possible reasons for the aberrations [16]. Ghazani *et al.* showed aberrant changes in array-CGH profiles of cultured cells fixed for 30min, 20hr and 1 week [11]. It was concluded that samples fixed for 20 hr showed similar, but notably not identical, profiles to freshly harvested DNA. Although the above reports cited discordance between array-CGH profiles of FFPE and fresh-frozen tissue, the issue of false positive target discovery was not mentioned, nor were alternative tissue fixation or embedding processes suggested.

Tissue processing by formalin-fixation followed by paraffin embedding provides an opportunity to store large banks of tissue and allows retrospective genomic studies of tumours to be performed, in which chromosomal imbalances associated with tumour development and progression may be identified. In contrast, the creation and hosting of biobanks of fresh-frozen tissue comes with limitations such as storage space and power requirements.

Currently in the clinical setting, emphasis is based on preservation of tissue morphology for pathological diagnosis, rather than the preservation of nucleic acids within tissue specimens. 10% buffered formalin, an aqueous dilution of formaldehyde, is the most widely used fixative as it preserves a wide range of tissues and tissue components and is inexpensive [17,18] . However, formaldehyde is known to interact with DNA, and initiate DNA degradation [19]. Numerous additional factors can also influence the recovery of high-quality DNA from tissues. Longer prefixation times, or time from surgery to fixation, can have detrimental effects on DNA integrity [20]. Although it has been reported that limited tissue fixation times

have minimal impact on array-CGH profiles [11], in the hospital laboratory, standard operating procedures can vary between hospitals and routine fixation times can vary from 6-48 hr.

Numerous alternative fixatives to NBF have, however, been reported to have minimal impact of nucleic acids while maintaining tissue morphology for diagnosis [21-27]. Alcohols such as ethanol and methanol have been shown to preserve high-quality RNA and DNA. However, alcohols are often not sufficient to maintain morphology for diagnosis *(21, 25, 26)*. The AMeX method, which is based on cold acetone fixation followed by clearing with methyl benzoate and xylene, then embedding in paraffin, has been shown to be a versatile multipurpose tissue-processing procedure, maintaining tissue morphology and DNA integrity (23). This method does, however, require longer fixation at colder temperatures than formalin fixation, but could be easily implemented in the clinical setting.

The HOPE-technique (Hepes-Glutamic acid buffer mediated Organic solvent Protection Effect) utilises a protection solution comprised of the organic buffer acetone, as the only dehydrating agent, and pure paraffin of 52-54 °C melting temperature. HOPE-fixed sections have been shown to exhibit formalin-like morphology (22) and provide an excellent preservation of proteins and nucleic acids (DNA and RNA) (28). High-quality DNA and RNA have been extracted from HOPE-fixed specimens up to 5 years after fixation (22). High-quality DNA suitable for use in array-CGH profiling experiments can successfully be extracted from tissues fixed using this method (supplementary figures 1 and 2). The HOPE technique, although marginally more time-consuming may therefore be important in enabling clinical diagnosis, while allowing for fundamental retrospective research studies.

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To conclude, we suggest that the use of suitable high-quality DNA will improve our confidence in identifying key genetic alterations in disease states such as cancer. Advances in DNA extraction methods and array-CGH protocols may increase the reliability of DNA profiles acquired from FFPE material, and implementation of new, multi-purpose fixatives may further improve the quality and suitability of DNA extracted from fixed tissue specimen while maintaining morphology for diagnosis.

Acknowledgements

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Figures

Figure 1

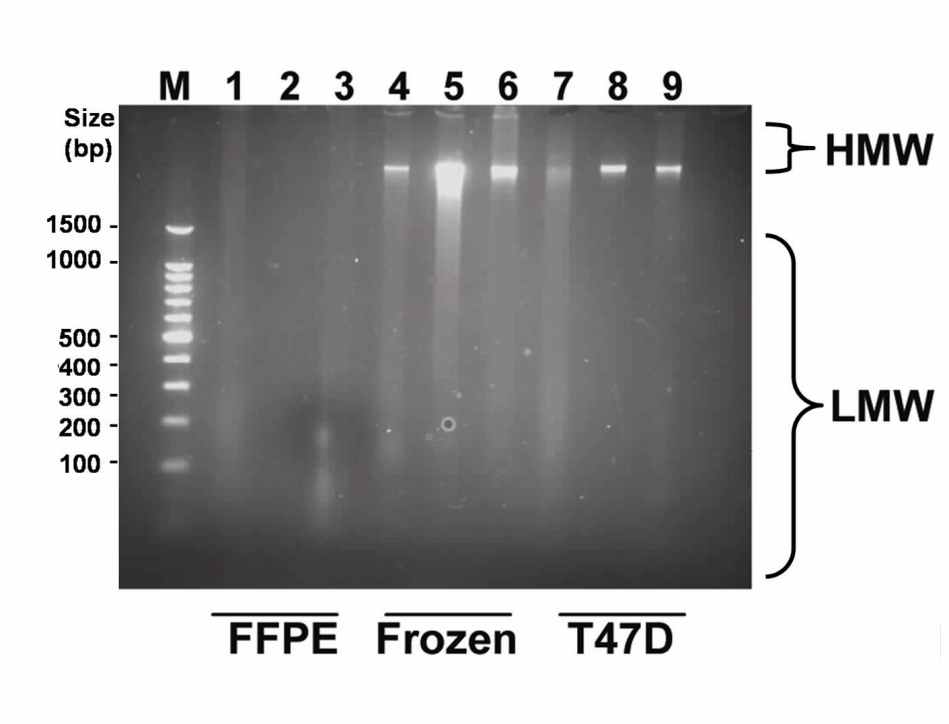


Figure 2

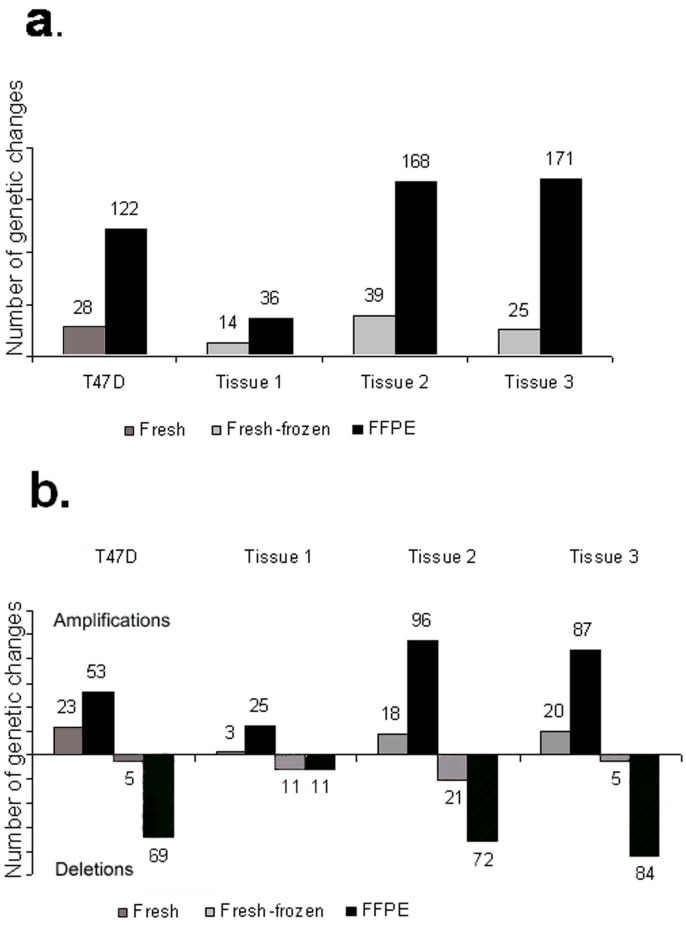

a.
Number of genetic changes
122
28
36
14
168
39
171
25
T47D
Tissue 1
Tissue 2
Tissue 3
Fresh
Fresh-frozen
FFPE
b.
T47D
Tissue 1
Tissue 2
Tissue 3
Amplifications
Deletions
Number of genetic changes
53
23
5
69
25
3
11
11
96
18
21
72
87
20
5
84
Fresh
Fresh-frozen
FFPE

Figure 3

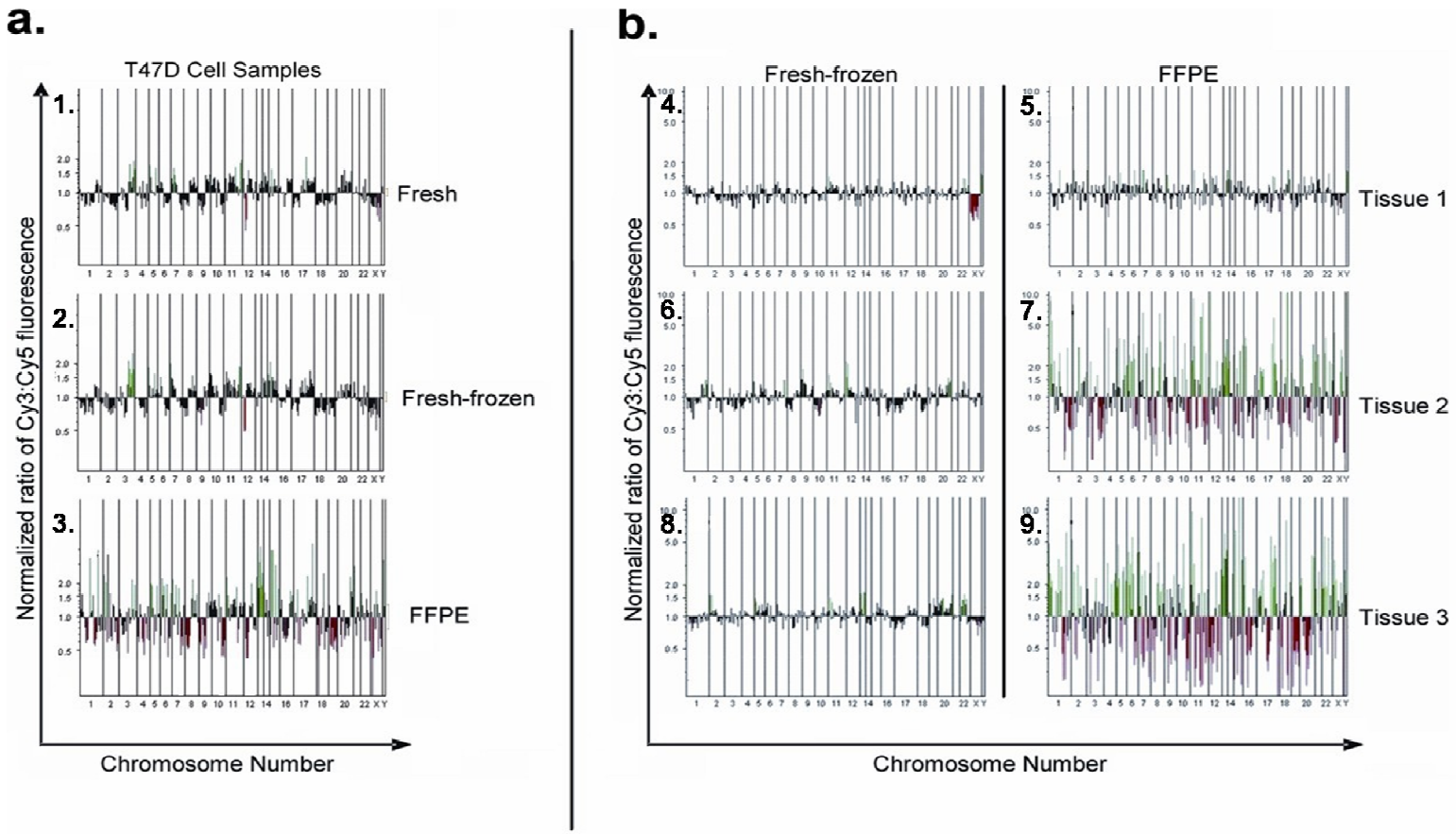

a.
T47D Cell Samples
1.
Fresh
2.
Fresh-frozen
3.
FFPE
Normalized ratio of Cy3:Cy5 fluorescence
Chromosome Number
b.
Fresh-frozen
FFPE
4.
5.
6.
7.
8.
9.
Tissue 1
Tissue 2
Tissue 3
Normalized ratio of Cy3:Cy5 fluorescence
Chromosome Number

Figure Legends

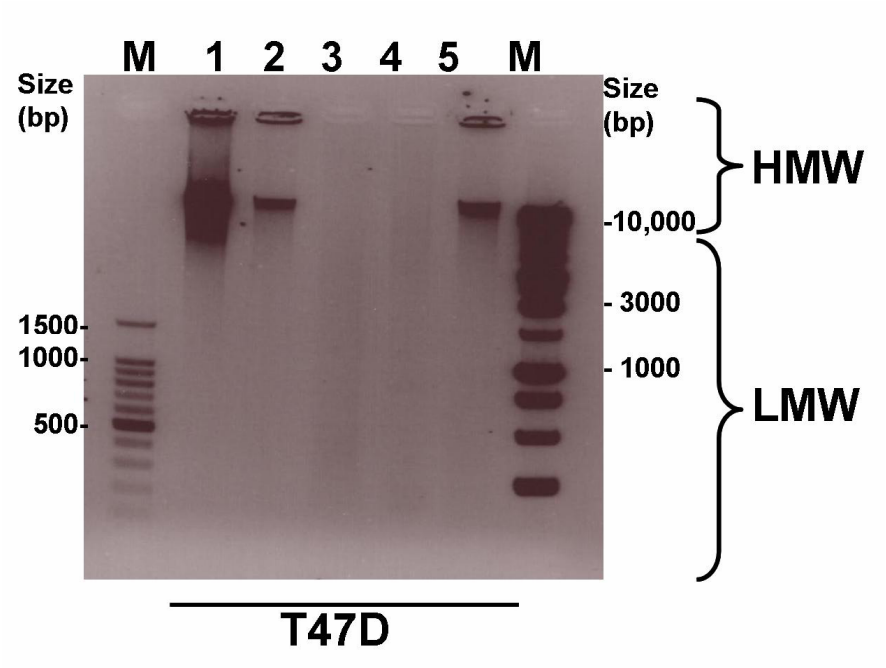
Figure 1. **Assessment of genomic DNA integrity via agarose gel electrophoresis.** Genomic DNA (100ng) extracted from cell lines and clinical tissue, with varying degrees of degradation is shown. High molecular weight (HMW) and low molecular weight (LMW) DNA are indicated. 100bp or 1kb DNA ladders from Promega were used as DNA size markers (M).
a. Lanes 1-3: DNA extracted from FFPE clinical tissues. **Lanes 4-6**: DNA extracted from fresh-frozen clinical tissues. **Lane 7**: DNA extracted from FFPE T47D cell line sample. **Lane 8**: DNA extracted from fresh T47D cell line sample. **Lane 9**: DNA extracted from snap-frozen O.C.T.-embedded T47D cell line sample.

Figure 2**. Numbers of genetic changes detected in DNA from matched FFPE and fresh or fresh-frozen samples using array-CGH.**
a. Total number of statistically relevant genetic changes detected in matched T47D cell line samples and matched clinical samples.
b. Number of DNA amplifications versus deletions detected in matched T47D cell line samples and matched clinical samples.

Figure 3. **Array-CGH profiles of fresh-frozen and FFPE tissue DNA.**
a. Array-CGH spectral profiles of differentially-processed T47D cancer cell pellets. **1**. Fresh T47D cell line DNA. **2**. Snap-frozen O.C.T. embedded T47D cell line DNA. **3**. FFPE T47D cell line DNA.
b. Array-CGH spectral profiles of matched fresh-frozen and FFPE DNA extracted from colon cancer clinical samples. **4**. Tissue 1 fresh-frozen DNA. **5**. Tissue 1 FFPE DNA. **6**. Tissue 2 fresh-frozen DNA. **7**. Tissue 2 FFPE DNA. **8**. Tissue 3 fresh-frozen DNA. **9**. Tissue 3 FFPE DNA.
Green: Statistically-significant genetic amplifications, Red: Statistically-significant genetic deletions.

Supplementary Figures

Supplementary Figure 1



Supplementary Figure 2

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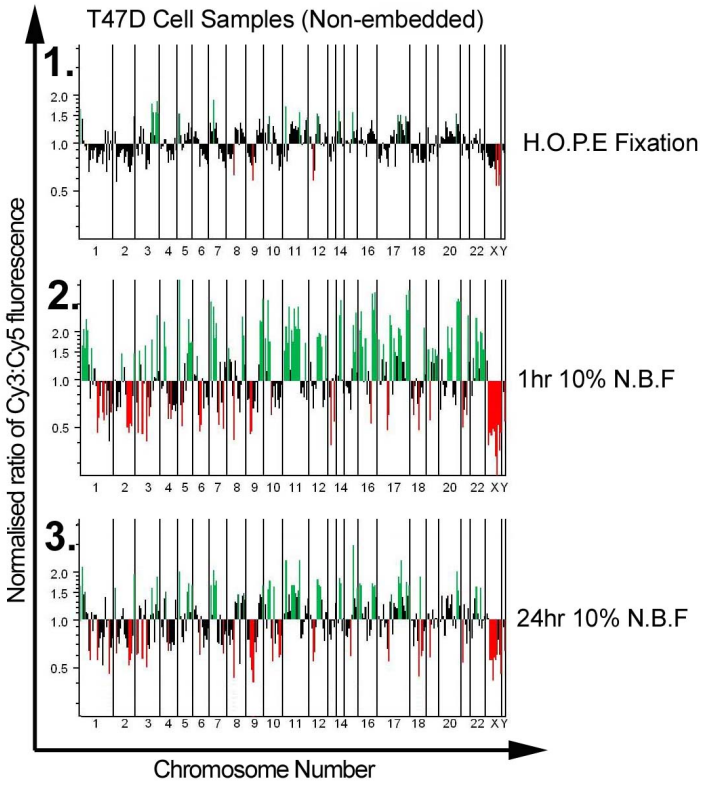

Supplementary Figure Legends

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Supplementary Figure 1. **Assessment of genomic DNA integrity via agarose gel electrophoresis**

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Lane 1: DNA extracted from fresh T47D pellet. **Lane 2**: DNA extracted from snap-frozen O.C.T.-embedded T47D cell line sample. **Lane 3**: DNA extracted from T47D pellet fixed for 1hr in 10% NBF. **Lane 4**: DNA extracted from T47D pellet fixed for 24hr in 10% NBF. **Lane 5**: DNA extracted from HOPE-fixed T47D pellet.

Supplementary Figure 2. **Array-CGH profiles of DNA non-embedded differentially fixed T47D pellets.**

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1. Fresh T47D cell line DNA for comparison. **2**. DNA extracted from HOPE-fixed T47D cell pellet. **3**. DNA extracted from 1hr 10%NBF-fixed T47D cell pellet. **4**. DNA extracted from 24hr 10%NBF-fixed T47D cell pellet.

Green: Statistically-significant genetic amplifications, Red: Statistically-significant genetic deletions.